In vitro screening of selected elite clones of *Eucalyptus tereticornis* Sm. for drought stress

A

Dissertation Report

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In

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CERTIFICATE

This is to certify that dissertation entitled "***In vitro* screening of selected elite clones of *Eucalyptus tereticornis* Sm. for drought stress"** submitted by Miss. Sukhmani Kaur (Roll no. 301701029) in the partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala. She has fulfilled all the requirements in completing this work under my supervision and guidance.

The matter embodied in this thesis has not been submitted in part or full to any other institute or university for the award of any degree or diploma.

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DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled **"*In vitro* screening of selected elite clones of *Eucalyptus tereticornis* Sm. for drought stress"** by the undersigned in the partial fulfilment of the requirement for the award of the degree of master of science in biotechnology, Thapar Institute of Engineering and Technology, Patiala is true and original record of my own independent and original research work carried out under the supervision of Dr. Anil Kumar, Associate Professor, Department of Biotechnology and Environment Sciences, Thapar Institute of Engineering and Technology, Patiala, India, this matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

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Abbreviations

%	Percentage
µl	Microlitre
µmol g^{-1}	Micromolar per gram
APX	Ascorbate peroxidase
BA	Benzyl adenine
CAT	Catalase
DMRT	Duncan's multiple range test
FW	Fresh weight
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
MDA	Malondialdehyde
Min	Minutes
mM	Millimolar
MS	Murashige and Skoog
NAA	α-naphthaleneacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$O_2^{\cdot -}$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
PEG	Polyethylene glycol
PGR	Plant growth regulator
POX	Peroxidase
PPO	Polyphenol oxidase
ROS	Reactive oxygen species
RWC	Relative water content
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TSS	Total soluble sugar
v/v	Volume by volume
w/v	Weight by volume

Summary

In the present study, *in vitro* screening of the four selected elite clones ('KE8', 'CE2', 'T1' and 'Y8') of *Eucalyptus tereticornis* for water stress (drought) tolerance was investigated. The proliferating multiple shoots were cultured on basal MS medium supplemented with 2.5 µM Benzyl adenine , 0.5 µM napthaleneacetic acid and different concentrations of mannitol (0-1000 mM). The shoot culture index of these clones was calculated under different level of stress and it was observed that shoot culture growth index of clone 'KE8' (141.42 %) was significantly higher than clone 'Y8' (106.07 %) on medium containing 1000 mM mannitol.

The tolerant clone 'KE8' exhibited 72.79 % relative water content on medium containing 1000 mM mannitol whereas it was reduced to 60.29 % in sensitive clone 'Y8' on the same medium after a culture period of 28 days. It was also recorded that the sap of drought tolerant clone 'KE8' had a osmotic potential of -3.30 MPa, whereas sensitive clone 'Y8' had a -3.79 MPa on medium containing 1000 mM mannitol which had water potential of -3.16 MPa.

In clone 'KE8', total chlorophyll and carotenoid contents of 116.88 µg g^{-1} fresh weight (FW) and carotenoid content of 15.671 µg g^{-1} FW were recorded on medium containing 1000 mM mannitol. However, significantly lower values for total chlorophyll (58.29 µg g^{-1} FW) and carotenoid contents (10.17 µg g^{-1} FW) were recorded for sensitive clone 'Y8' on the same medium and after the same culture period.

Maximum proline accumulation (4 µmol g^{-1} FW) was observed for tolerant clone 'KE8' on medium containing 750 mM mannitol whereas, on the same medium and after same culture period, clone 'Y8' accumulated 3.2 µmol g^{-1} FW of proline . Maximum reducing sugars content (130.32 mg g^{-1} DW) was recorded in the cultures of clone 'KE8' on medium containing 1000 mM mannitol and on same medium drought sensitive clone 'Y8' accumulated 79.09 mg g^{-1} DW of reducing sugar. Similarly, total soluble sugar content also increased significantly with increasing concentration of mannitol, increase was more pronounced in tolerant clone than sensitive clone 'Y8'.

Increased antioxidant enzyme activity played a significant role in imparting drought stress tolerance in various clones of *E. tereticornis.* In this context, maximum superoxide

dismutase (SOD) activity (11.09 U mg^{-1} protein) was recorded in the cultures of clone ‘KE8’ on medium containing 1000 mM mannitol, whereas significantly lower SOD activity (6.99 U mg^{-1} protein) was recorded in the cultures of clone ‘Y8’. Further, the activities of other antioxidant enzymes such as catalase, peroxidase and ascorbate peroxidase were also studied and it was observed that the activities increased significantly with increasing concentration of mannitol. The sensitive clone ‘Y8’ also recorded higher levels of malondialdehyde content (21.06 µmol g^{-1} FW) on medium containing 1000 mM mannitol, indicating the extent of lipid peroxidation of cellular membrane due to the formation of reactive oxygen species.

The present study explored the effect of mannitol on selected elite clones of *Eucalyptus tereticornis* for drought tolerance and studied various physiological and biochemical parameters to associate their role with drought tolerance. Taken together, the study resulted in the identification of tolerant clone ‘KE8’ which can be easily grown in areas with limited rainfalls.

1. Introduction

India's forest cover is declining rapidly due to climatic changes and related environmental stresses (Anderegg et al. 2013; Millar et al. 2007). Forest trees are routinely exposed to various environmental stresses such as salinity, drought, heavy metal toxicity etc. adversely affecting the productivity worldwide (Nagarajan and Nagarajan 2009). Drought is considered as one of the major factor limiting the tree growth under natural environments (Fang and Xiong 2015). Depending upon the severity and duration of the drought, various morphological and physiological adjustments associated with cellular, molecular and organ levels takes place (Chaves et al. 2003; Reddy et al. 2004). In the long run, tree species have developed series of mechanisms to adopt drought conditions such as root architecture, restricted growth and an increase in water use efficiency (Farooq et al. 2009). The frequency and distribution of drought may increase in the near future, and so does their effect on forest (Allen et al. 2010). Hence, it is of paramount importance to assess and screen the drought tolerant varieties with respect to growth performance and production for plantation under water deficit conditions. The screening of drought tolerant varieties depends upon response of a plant after exposure to different levels of stress. The tolerance can be assessed in terms of plant development, physiological and biochemical markers which had been extensively studied in past to understand the mechanism behind drought tolerance (Hellal et al. 2018).

Drought is characterized by limited rainfall, non availability of water for irrigation, and reduced soil water (Kijne 2006). Worldwide, due to reduced rainfall and reciting ground water, the percentage of drought affected areas increased considerably in the past. However, there has been limited progress in the development of drought tolerant clones, especially in woody species (Rai et al. 2011; Watanabe et al. 2000). Therefore, screening of *Eucalyptus tereticornis* could serve as an important alternative for effective reforestation with limited water resources. Further, field based screening for drought tolerance is very slow and limited studies can be performed within in a short period of time (Bernier et al. 2008; Cattivelli et al. 2008; Ludlow and Muchow 1990). Therefore, use of plant tissue culture have gained significant attention for screening of drought using different stress inducing agents like, sodium chloride, sorbitol, mannitol and polyethylene glycol (PEG) (Ahmad et al. 2007; Gopal and Iwama 2007; Sadeghian and Yavari

2004). The effectiveness of these have been already studied in screening salt tolerant varieties using sodium chloride as stress inducing agent in *E. tereticornis* (Singh and Kumar, unpublished). The use of various water stress inducing agents like sorbitol, mannitol and PEG had been demonstrated in case of other plant species for screening tolerant clones under *in vitro* conditions (Errabii et al. 2006; Gopal and Iwama 2007)

In order to identify superior clones tolerant to water stress, it is important to understand the mechanism behind stress tolerance (Tuteja 2007). Water stress is portrayed by the reduction in osmotic potential along with various physiological and biochemical changes (Ahmad et al. 2007; Shao et al. 2008). To maintain the osmotic balance under stress conditions, various compatible solutes such as proline, glycine are accumulated at higher concentrations in cytosol and extracellular matrices (Ashraf and Foolad 2007). However, plant response gets intensified depending upon the type and level of water stress. During stress, overproduction of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide radical, singlet oxygen and hydroxyl ions also aggravate the adverse influence generated due to osmotic stress (Gill and Tuteja 2010). Various antioxidant enzymes participate in the detoxification of H_2O_2 to water involving, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase, guaiacol peroxidase, glutathione peroxidase etc. (Hellal et al. 2018; Rao and Jabeen 2013). Along with, non-enzymatic antioxidants such as ascorbate, carotenoids, polyphenols and flavonoids etc. prevents the formation of ROS by interacting with lipids to stabilize cellular membranes (Ashraf 2009). However, uncontrolled production of toxic ions such as singlet oxygen, hydroxyl ions lead to membrane lipid peroxidation (Rao and Jabeen 2013; Reddy et al. 2004). Further, the drought not only causes alteration in photosynthesis, but it also reduces the ion uptake, carbohydrates, and nutrients from the soil (Arzani 2008; Hu and Schmidhalter 2005). As a consequence, various physiological, biochemical and molecular traits contribute to stress acquisition in plants (Hellal et al. 2018; Monneveux et al. 2013).

Australian tree, *Eucalyptus* had been introduced in India for the conservation of forest from the past few decades. In India, *E. tereticornis* is widely grown for trading purposes due to its commercial utilization for pulp and paper industry, uses in furniture, fuelwood, charcoal and eucalypt oils (Aggarwal et al. 2010). Therefore, there is need to increase its plantation in areas

where rainfall or irrigation supply is limited. However, identification of tolerant clones is laborious task if performed under field conditions. Alternatively, screening and identification through plant tissue culture is effective and economical. Taking this into consideration, the present study is focused on the following key points:

i. To study the effect of drought stress inducing agent(s) on growth and morphology of selected elite clones of *Eucalyptus tereticornis*
ii. To examine various physiological and biochemical parameters associated with drought stress tolerance

2. Review of literature

Plants experience stress due to lack or excess of an essential component(s) required for normal growth or metabolism (Alam 1999; Ashraf 2009). They generally face these two situations simultaneously. Numerous types of stresses including both biotic and abiotic have been anticipated in the recent years, which proved to be detrimental for the growth and yield of crops under natural environments (Gill and Tuteja 2010; Mittler 2006; Suzuki et al. 2014). Drought stress have been long recognized as the major abiotic stress restraining the productivity across various parts of the world (Hamayun et al. 2010). Drought stress is rising rapidly in areas due to increasing soil salinity, limited water supply and erratic rainfalls over an extended periods, which ultimately results in the death of plant (Salehi-Lisar and Bakhshayeshan-Agdam 2016). Inadequate rainfall is found to be major reason of drought stress because in 70 % of the areas, cultivation of various crops is under rain fed conditions (Shekafandeh and Hojati 2012).

To counteract drought stress, various investigations have been done in the past in different plant genera such potato (Gopal and Iwama 2007), sugarcane (Rao and Jabeen 2013), wheat (Ji et al. 2014a) etc. However, drought stress tolerance is a complex mechanism involving various metabolic pathways and manipulation of gene(s) at the molecular level (Hellal et al. 2018; Valliyodan and Nguyen 2006). Therefore, understanding of the plant response is required for the selection of drought resistant plants. In the present study, we identified *Eucalyptus tereticornis*, commercially important tree, grown in various states of India. The importance of *Eucalyptus* had been known due to its different therapeutic properties as well as acts as an important source of fuel wood (Vecchio et al. 2016). It was also reported that eucalyptus oil extracted from leaves possess antiseptic, anti-inflammatory properties (Dhakad et al. 2018). Based on the clonal difference, there is difference in traits of wood and *E. tereticornis* has been selected as important source of wood for pulp and paper industries (Aggarwal et al. 2010; Chauhan and Aggarwal 2011). The alarming situation of drought conditions is posing threat to its cultivation thus hampering supply of raw material to pulpwood industry. Therefore, there is need to select the tolerant clone(s) in *E. tereticornis* for meeting the rising demands. For the selection of tolerant clone(s), it is a prerequisite to understand the various physiological and biochemical changes which contribute to withstand the stress conditions.

2.1 *In vitro* screening for drought stress

In vitro screening offers potential advantages over *in vivo* screening such as larger population can be screened simultaneously, independent of extensive labour requirements and can be performed throughout the year within a time interval (Rao and Jabeen 2013). Further, for *in vitro* screening, micropropagation protocol had been already established for the selected elite clone(s) of *E. tereticornis* (Aggarwal et al. 2010). The use of various stress inducing agents such as sorbitol, mannitol or PEG had been reported to maintain the similar conditions to the dry soil without interfering with other medium constituents (Goodarzian Ghahfarokhi et al. 2015). Therefore, the presence of different stress inducing agent(s) and their concentration in the medium will determine the relative tolerance of plants to drought stress. Sorbitol or mannitol, being non-ionic and penetrating, are widely used as osmotic agents for the selection of drought tolerant varieties in potato (Gopal and Iwama 2007). The use of PEG is also preferred due to its high molecular weight and its non penetration into the cells (Gopal and Iwama 2007). At the same osmotic potential, PEG showed more inhibitory action when compared with other stress inducing agent like sorbitol. Thus, screening of tolerant clone(s) is a tremendous task involving the use of various stress inducing agents and determination of the inhibitory concentration for the selection of sensitive and tolerant clones.

2.2 Physiological parameters

Drought is considered as one of the major threat in reducing the plant growth and adversely affecting production. It is evident from various studies that drought had negative impact on various growth attributes which can be due to reduced cell division and/or elongation of meristematic cells (Farooq et al. 2009; Hellal et al. 2018). The one of the primary effect is reduction in seed germination followed by seedling establishment. In this context, various studies had elucidated the fact that when exposed to water deficit conditions, germination percentage rate and plumule length reduced significantly (Hellal et al .2016(Duman 2006). One of the most sensitive step to water stress is germination and it becomes important in water deficient areas to undergo modification of traits related to germination so that they are able to survive under water

deficiency (Wani et al. 2010). Leaf area and expansion is also reduced limiting the contents of photosynthetic pigments and ability to absorb water. It was also revealed that root and shoot weight of plants gradually reduced when exposed to increasing concentration of PEG in case of potato (Gopal and Iwama 2007) and wheat (Baalbaki et al. 1999). Many studies also emphasized that under water stress conditions, various parameters like shoot and root lengths, germination rate, shoot and root weight were considered as the most effective traits for the selection of tolerant genotype(s) (El-Denary and El-Shawy 2016; Hellal et al. 2018; Sorkheh et al. 2011).

2.3 Relative water content

The relative water content (RWC) is a measure of dehydration tolerance level in plants (Sinclair and Ludlow 1986). Increase in drought stress resulted in decline of RWC in rice leaves (Hsu and Kao 2003), pigeon pea plants (Kumar et al. 2011) and barley (Yuan et al. 2005). Highest and lowest value of RWC was recorded in medium supplemented with and without 20 % PEG, summarizing the fact that drought reduces the ability to absorb water up to an extent that is insufficient for normal growth (Hellal et al. 2018). PEG induced drought conditions decreased the RWC of the shoots, but in case of barley (Hellal et al. 2018), water status of the shoots had been dependent on the size of the seeds. Seedlings from larger seeds had higher tissue water content than that of smaller seeds because they have longer root length and higher water uptake ability. (Hellal et al. 2018; Luis et al. 1992). Further, the RWC was found to be different in various plant parts such as roots and leaves under drought conditions (Shivakrishna et al. 2018). It had been reported in other crops such as tomato (Zgallaï et al. 2006) and leaves of peanut plant (Shivakrishna et al. 2018), where RWC decreased at higher stress level.

2.4 Osmotic potential

Stress inducing agents such as mannitol, sorbitol and PEG reduced the water potential of culture medium. They cause inhibitory effect on cultural explants and prevent them to take nutrients and water from the cultural medium. In response to osmotic imbalance, plants accumulate organic compounds such proline, glycine, sugar and amino acids in the cytoplasm of plant cells (Ackerson 1981; Morgan 1992). Their accumulation reduced the osmotic potential of the plants cells and considered as an important adaptive response which maintain the turgor

pressure of plant cells and growth of the plants (Bajji et al. 2000). Decrease in osmotic potential to maintain the turgidity in the leaves of plants had been reported earlier in woody genera including *Acacia* (Nabil and Coudret 1995). Osmotic imbalance in culture medium induced by mannitol reduced the dry matter accumulation in different plants such as wheat, potato and rapeseeds (Lipavska and Vreugdenhil 1996).

2.5 Chlorophyll content

Degradation of chlorophyll content is a common phenomenon in plants as leaf development is sensitive to dehydration. It was observed that water deficiency had a negative impact on the pigment contents in field grown plants (Farooq et al. 2009). Dehydration leads to the 48 % reduction in chlorophyll contents (chlorophyll a and b) in peanut leaves when compared to the control (Shivakrishna et al. 2018). Moreover, less reduction of chlorophyll B content was observed than chlorophyll A in sesame plants when cultured on MS medium containing different concentrations of PEG (Hsu and Kao 2003). In addition, carotenoids also played an important role in antioxidants defense system during stress conditions (Jaleel et al. 2009).

2.6 Proline accumulation

Drought stress triggers the accumulation of various amino acids such as proline, glycine, alanine, arginine etc. by several folds for the scavenging of ROS, stabilizing cell membrane and maintenance of cellular osmotic potential (Errabii et al. 2006; Rai et al. 2011; Slama et al. 2015) Proline accumulation was considered as the basis for the selection of tolerant plants in groundnut, Indian mustard, coconut, sugarcane (Rai et al. 2011). Moreover, the tolerant callus of sugarcane also accumulated more amount of proline than non-tolerant callus (Rao and Jabeen 2013). It was also postulated that proline accumulation act as an good indicator for selection of water stress tolerant clones in sweet cherry (Sivritepe et al. 2008) and apple (Sotiropoulos et al. 2006). Proline accumulation increased by ~5 folds in barley roots when exposed to PEG-8000 indicating its important role in premature differentiation of root apical region under stress conditions (Ji et al. 2014b).

2.7 Total Soluble sugar content

The accumulation of various osmoprotectants such as sugars protect the plants from the deleterious effects of oxidative stress has been recorded (Bohnert and Shen 1998). Under drought conditions, soluble sugar content increased with the increasing concentrations of PEG in culture medium. It gets accumulated significantly in the leaves of the fig cultivars and significantly increased under stress (Shekafandeh and Hojati 2012). It was reported that under water stress conditions, sugar accumulation occurred due to starch hydrolysis therefore, the amount of starch decreases with increasing sugar content (Shawky et al. 1996). The accumulation of various sugars such as sucrose, trehalose etc. act as an osmoticum and provide stability to cellular membrane (Slama et al. 2015). Sugar accumulation had been well studied under saline conditions in crops such as chickpea varieties (Mafakheri et al. 2010) and almonds seedling (Zamani et al. 2001) as well as under *in vitro* induced salt stress conditions in date palm (Al-Khayri and Al-Bahrany 2004; Sivritepe et al. 2008).

2.8 Antioxidant enzyme activities

Water deficiency causes damage of the various cellular structures and malfunctioning of the metabolic activities (Karimi et al. 2012). Drought conditions also stimulated the formation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·). ROS production is localized to various cellular components such as mitochondria, peroxisomes and chloroplasts of the cells (Das and Roychoudhury 2014). The uncontrolled production of ROS under stress conditions caused extensive damage to proteins, lipids and other biomolecules (Patade et al. 2012). However, plants have innate ability to protect themselves at sub cellular level from toxicity of free radicals by inducing the antioxidant activity. This antioxidant mechanism lead to the activation of free radicals scavengers like glutathione, ascorbate and other antioxidant enzymes like SOD, POX, GPX (Hellal et al. 2018). In response to abiotic stress, SOD acts as first line of defense involved in the dismutation of $O_2^{\cdot -}$ to H_2O_2 (Das and Roychoudhury 2014). Further, the various other antioxidant enzymes catalysis the dismutation of H_2O_2 to H_2O and O_2 without involving any reductant such as catalase (CAT) and with the help of reductants like APX utilizes ascorbic acid and ortho-diansidine is used by POX enzyme. It was reported that selected callus of sugarcane

under drought conditions led to significant increase in the activity of these antioxidant enzymes (Rao and Jabeen 2013). However at higher concentration of PEG, antioxidants activities of non–selected callus declined significantly than that of selected callus (Rao and Jabeen 2013). Various other reports in different plant species also highlighted the increase in activity of these antioxidant enzymes with increasing drought stress (Hellal et al. 2018; Hsu and Kao 2003; Kaya et al. 2013; Rao and Jabeen 2013).

2.9 Lipid peroxidation

The liberation of ROS causes oxidative stress damage to lipid membrane and degradation of proteins (Anjum et al. 2011). This structural damage of membrane is called as lipid peroxidation, is determined in terms of malondialdehyde (MDA) as by product. MDA forms a red colour complex with thiobarbituric acid (TBA), which can be quantified spectrophotometrically. Higher MDA content was recorded in sensitive callus lines of sugarcane compared to PEG tolerant lines, which indicates that tolerant clones maintained membrane integrity and helped in normal growth under stress conditions (Rao and Jabeen 2013). Lipid peroxidation induced by PEG, NaCl was also reported in blueberry (Patade et al. 2012) and beans (Yasar et al. 2010).

Table 2.1: *In vitro* screening of different plants for drought stress

Plant species	Medium composition	Explant	Drought stress inducing agent	Response	References
Barley (Giza-123,124,125,126,127,129,130,134,135,2000)	Distilled water	Seeds	PEG 6000 (0-20 % w/v)	¶ High germination rate (>76 % at 10 % w/v PEG) was observed in cultivars Giza 127, 134 while low germination rate was observed for other cultivars ¶ High antioxidant enzyme activities for cultivars (Giza 126, 127, 134, 136 and 2000) at 10 % w/v PEG concentration	(Hellal et al. 2018)
Wheat	MS	Seeds	PEG 8000 (5 % w/v) for 8 h and 72 h	¶ Accumulation of proline increased by ~5 folds and soluble sugar by 2 folds after 72 h stress treatment	(Ji et al. 2014b)
Lentil (Castelluccio, Eston, Pantelleria, and Ustica)	Distilled Water	Seeds	PEG 6000 (10-21 % w/v)	¶ Reduction in germination and water content with increasing concentrations of PEG ¶ Decrease in α-amylase and α-glucosidase activities affecting sensitive cultivars (Pantelleria and Ustica) more than the tolerant ones.	(Muscolo et al. 2014)
Maize	Hoagland's Nutrient Solution	Surface disinfected seeds	NaCl (100 mM) Mannitol (15 and 30 mM) Thiourea (3.5 and 7 mM)	¶ Growth reduction increase in the activities of CAT, SOD and polyphenol oxidase (PPO) with the application of salt stress ¶ Addition of mannitol or thiourea resulted in salt stressed plants lead to reduction in the activities of CAT, SOD, POX and PPO	(Kaya et al. 2013)

				¶ Mannitol lead to improved salt tolerance in maize in terms of growth and physiological attributes	
Sugarcane	MS+ 4.5 µM 2,4 D + 2.32 µM Kinetin	Calli	PEG 6000 (0-30 % w/v)	¶ Decrease in fresh weight of selected calli by 30 % at high PEG concentration ¶ 10 fold increase in proline accumulation at 20 % w/v PEG ¶ Increase of antioxidant enzyme activities by several folds till 15 % w/v PEG ¶ MDA contents increased by 26.32 %	(Rao and Jabeen 2013)
Rice	MS	Calli	PEG-6000 (1-7 % w/v)	¶ Minimum (21 %) and maximum (3 %) decline in calli volume by was observed in Narendra 359 and Pusa Basmati 1 ¶ proline accumulation increased by 1,570.7 % in Narendra 359 at 7 % w/v PEG ¶ Taraori Basmati hold higher percentage increase in total chlorophyll and chlorophyll a/b ratio	(Joshi et al. 2011)
Almonds (*Prunus* L. spp.)	WPM	Surface disinfected seeds	PEG -8000 (3, 6, 9, 12 mM) Sorbitol (100, 200, 300, 400 mM)	¶ Differential response of various species in terms of reduction in plant height, plant fresh weight, root length, root dry weight, root volume was observed at different concentrations of sorbitol and PEG	(Sorkheh et al. 2011)
Soyabean (Indian and	MS+ 4.5 µM 2,4 D + 2.32	Calli	PEG 6000 (4-6 % w/v)	¶ Reduction of calli growth and shoot induction in Indian and Bulgarian	(Sakthivelu et al. 2008)

Bulgarian cultivars)	µM Kinetin			cultivars ¶ Decline in osmotic potential of callus tissues in both cultivars	
Potato (IWA -1, IWA-2,IWA-3)	MS	Sprouted microtubers	PEG-8000 (3, 6, 9, 12 mM) Sorbitol (100-400 mM)	¶ Based on various growth parameters, IWA-1 found to have more drought tolerance ability than IWA-2 and 3 at 200 mM sorbitol and 3 mM PEG -8000	(Gopal and Iwama 2007)
Sugarcane (R570, CP59-73 and NCo310)	MS	Calli	NaCl (50-150 mM) Mannitol (100-300 mM)	¶ Decline of relative growth rate when exposed to high concentrations of NaCl and mannitol affecting sensitive cultivar (CP59-73) more than the other two cultivars ¶ 13 fold increase in proline accumulation in CP59-73 cultivar and 10 fold in both NCo310 and R570 cultivars under mannitol induced drought conditions	(Errabii et al. 2006)
Rice	Distilled water	Leaves	PEG-6000 and Sorbitol (-1.5 MPa)	¶ Decrease in relative water content and increase in MDA contents when exposed to PEG-6000 for 12 h ¶ Sorbitol treatment resulted in greater increase in the activities of POX, APX & GR than PEG-6000 treatment	(Hsu and Kao 2003)
Mexican marigold (P2, P6, P8, PM1, PM2,PM3,P4)	½ MS+ 2.85 µM IAA	Surface disinfected nodes	Mannitol (0-100 mM)	¶ Nodal segments of PM3 tolerated upto till 60 mM mannitol for 4 weeks ¶ Increase in proline and sugar accumulation at 30 mM mannitol	(Mohamed et al. 2000)

				concentration in PM3 ¶ Stress tolerant cultivar PM3 tolerated a water potential of -0.178 MPa	
Populous	½ MS +0.05 µM NAA	Microshoots	Mannitol (0-400 mM) NaCl (0-250 mM)	¶ 100 % survival rate for *P. euphratica* at all mannitol concentrations ¶ Proline accumulation was increased by 9 folds at 400 mM mannitol and 8 folds at 250 mM NaCl concentrations	(Watanabe et al. 2000)
Brassica juncea	MS +10.74 µM NAA + 13.32 µM BA	Calli	Mannitol (0-329 mM) NaCl (0-171 mM) PEG-6000 (0-330 mM)	¶ Declined calli growth with step wise increase in NaCl concentration ¶ Increased proline accumulation by 316 %, 211 % and 223 % at higher NaCl, mannitol and PEG-6000 concentrations	(Gangopadhyay et al. 1997)

2,4 D: 2, 4-Dichlorophenoxyacetic acid; BA: Benzyl adenine; IAA: Indole-3-acetic acid; MS: Murashige and Skoog medium; NAA: α-naphthalene acetic acid; NaCl: Sodium chloride ; PEG: Polyethylene glycol; and WPM: Woody plant culture.

3. Objectives

- ¶ To examine *in vitro* growth and morphogenesis of different clones of *Eucalyptus tereticornis* under mannitol induced drought conditions
- ¶ To study various physiological and biochemical parameters in different clones of *Eucalyptus tereticornis* vis-à-vis drought stress in culture
- ¶ To assay antioxidant enzyme activities of culture under drought stress conditions

4. Materials and Methods

4.1 Chemicals and glassware

All chemicals (AR grade) were purchased from Hi Media laboratories (Mumbai). The experiments were performed in 300 ml culture bottles (Kasablanka Corporation, Mumbai). Glassware like measuring cylinders, beakers etc. were purchased from Borosil Glass Works Ltd., Mumbai, India.

4.2 Medium preparation

For MS medium (MS, 1962 Detailed in Annexure I) preparation, the required quantities of concentrated stocks of different constituents such as macronutrients, micronutrients and vitamins were added and solution was mixed properly. Sucrose (3.0 % w/v) was also added to the medium. After mixing well, final volume was raised using distilled water, pH of medium was titrated to 5.8 using 0.1N KOH and/or 0.1N HCl. Thereafter 0.8 % (w/v) agar was added to each culture vessel. The medium was poured into these vessels (50 ml each). The mouth of culture vessels was closed with plastic lids and labelled. These vessels containing medium were autoclaved at temperature of 121 °C and pressure 15 psi for 20 minutes. Depending on the sensitivity of plant growth regulators (PGRs) to high temperature, PGRs were added before/after autoclaving the media to prevent their activity. After this the medium was allowed to cool and stored in dust free environment inside the culture room.

4.3 Plant material and culture conditions

Four clones (‘KE8’, ‘CE2’, ‘T1’ and ‘Y8’) of *E. tereticornis* were selected for the present study. Cultures were maintained on MS medium fortified with 2.5 µM 6-benzyl adenine (BA) and 0.5 µM α-naphthaleneacetic acid (NAA) (C55) for 4 weeks before experimentation (Aggarwal et al. 2010). The plantlets were maintained under the controlled culture conditions at 25±2°C Temperature, 70 % Relative Humidity, 16/8 h of light/dark period and 42 µmol m^{-2} s^{-1} Light intensity.

4.4 Drought tolerant screening

For drought stress treatment application, the proliferating multiple shoot clumps were cultured on C55 medium containing different concentrations of mannitol (250, 500, 750 and 1000 mM) for a culture period of 28 days. Culture growth index of various clones were calculated as follows:

$$\text{Culture growth index} = \frac{\text{Fresh shoot biomass recorded after 28 days}}{\text{Fresh shoot biomass inoculated in the beginning of experiment}} * 100$$

4.5 Relative Water Content (RWC)

The fresh weight (FW) of the tissue was taken and then it was saturated in distilled water till the constant turgid weight (TW) was obtained. Then, tissue was then dried in an oven (pre-heated to 80 ^{0}C) till the constant dry weight (DW) was achieved. RWC was calculated as follows (Smart and Bingham 1974)

$$\text{RWC} = \frac{\text{FW}-\text{DW}}{\text{TW}-\text{DW}} * 100$$

4.6 Osmotic potential

Fresh tissue sample (0.5 g FW) was placed in a syringe and quickly frozen at -20 ^{0}C. Osmotic potential was determined (Hernández-Sebastia et al. 1999) in vapour pressure osmometer (Wescor 5500, Logan, Utah, USA) from the 10 µl tissue sap collected after thawing of frozen samples. Van't Hoff empiral relationship was used to express osmotic potential in MPa.

4.7 Pigment contents

Leaf (0.2 g FW) from different tissue samples were extracted in 10 ml of 80 % w/v acetone for the determination of chlorophyll a, chlorophyll b, total chlorophyll, carotenoid contents. Absorbance at 480, 645 and 663 nm was recorded using UV-Vis spectrophotometer

(U-2900, Hitachi, Tokyo, Japan) (Arnon 1949). The net pigment contents were calculated using the equations written below:

Chlorophyll a (µg g^{-1} FW): [12.21*(Abs at 663 nm)-2.81*(Abs at 645 nm)]*50

Chlorophyll b (µg g^{-1} FW): [20.81*(Abs at 645 nm)-5.03*(Abs at 663 nm)]*50

Total chlorophyll content (µg g^{-1} FW): Chlorophyll a + Chlorophyll b

Carotenoid Content (µg g^{-1} FW): ([1000*(Abs at 480 nm)-3.27(Chlorophyll a)-104 (Chlorophyll b)]/229)*50

4.8 Proline Estimation

Ninhyrdin acid reagent was used to analyze proline content in different clones after exposure to drought stress (Bates et al. 1973). Fresh tissue (0.3 g FW) was extracted in aqueous sulfosalicylic acid. After centrifugation, it was allowed to react with equal volume of acid-ninhydrin solution and glacial acetic acid for 60 min in a water bath (100°C). The reaction mixture was then transferred to ice bath for termination. The addition of toluene to the reaction mixture, resulted in the separation of two layers. The absorbance of upper layer containing proline was measured by a UV-Vis spectrophotometer (U-2900, Hitachi, Tokyo, Japan) at 520 nm. The proline content was determined from the curve prepared using L-proline as standard and expressed as µmol g^{-1} FW.

4.9 Sugar estimation

Three composite samples (50 mg dried at 80 °C) were homogenized in 80 % aqueous ethanol and extracted for 3 hour. The extraction was performed twice, supernatant was collected and pooled to final volume of 25 ml. The supernatant was used for the estimation of total soluble sugars (TSS) (Dubois et al. 1956) and reducing sugars (RS) (Sumner and Howell 1935). For TSS estimation, reaction mixture consists of 500 µl extract, 10 µl phenol reagent (90 % v/v) and 5 ml concentrated sulphuric acid and 490 µl distilled water was added. The reaction mixture was incubated at room temperature for 30 min and absorbance was measured at 485 nm. The

concentrations were calculated using external curve prepared taking known concentrations of glucose.

For RS estimation, 1 ml dinitrosalicylic acid reagent was added to 500 µl extract and final volume (2 ml) was made with the help of distilled water. The contents of the reaction mixture were mixed and kept in a boiling water bath for 10 min. After cooling to room temperature, absorbance was recorded at 560 nm. The concentrations were calculated using the curve obtained from the known concentrations of glucose used as standard and expressed as mg g^{-1} DW.

4.10 Protein extraction

Fresh tissue (1 g FW) was homogenized in 50 mM potassium phosphate buffer (pH=6.8) supplemented with 100 mM sucrose, 5.7 mM cysteine hydrochloride, 0.2 g/l polyvinyl pyrrolidone. The homogenate was centrifuged and supernatant was pooled to a final volume of 10 ml. Equal volume (5 ml) of homogenate and 20 % (w/v) chilled trichloroacetic acid were mixed and incubated for 4 hour at 4 ^{0}C. The protein precipitates after dissolving in 3 ml of 1N NaOH were quantified by (Lowry et al. 1951). The remaining supernatant (5 ml) was used for the determination of various antioxidant enzyme activities.

4.11 Antioxidant enzymatic assays

The activity of antioxidant enzymes (superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase) was evaluated using standardized protocols with minor modifications and expressed in terms of U mg^{-1} protein.

4.11.1 Superoxide dismutase activity

Superoxide dismutase activity was estimated according to the methodology proposed by (Beauchamp and Fridovich 1971).

Table 4.1 Reaction mixture (3 ml) composition for SOD assay:

Reagents	Working concentrations	Volume (µl)
50 mM phosphate buffer	50 mM	2186
Nitro blue tetrazolium (NBT)-1 Mm	75 µM	225
1 M L. Methionine	13 mM	39
3.0 mM EDTA-disodium	100 µM	100
0.2 mM Riboflavin	2 µM	30
Extract		150

4.11.2 Catalase activity

For Catalase activity, the decrease in absorbance at 290 nm according to the method of (Aebi 1984).

Table 4.2 Reaction mixture (3 ml) composition for catalase assay:

Reagents	Volume (µl)
30 % v/v H_2O_2	50
50 mM Phosphate buffer	2850
Extract	100

4.11.3 Peroxidase activity

The increase in absorbance for peroxidase assay due to dehydrogenation of o-dinsidine was recorded at 470 nm (McEwen Jr 1971).

Table 4.3 Reaction mixture (3 ml) composition for peroxidase assay:

Reagents	Volume (µl)
Phosphate buffer- 50 mM	2750
O-dinisidine solution (1 % w/v)	100
1 % (v/v) H_2O_2	50
Extract	100

4.11.4 Ascorbate peroxidase activity

Ascorbate peroxidase activity was calculated as per the protocol described by (Nakano and Asada 1981). The decrease in absorbance due to the oxidation of ascorbate was recorded every 5 sec for 3 min at 290 nm.

Table 4.4 Reaction mixture (3 ml) composition for ascorbate peroxidase assay:

Reagents	Volume (µl)
Phosphate buffer- 50 mM	2875
100 mM Ascorbate	15
10 mM EDTA	30
30 % (v/v) H_2O_2	30
Extract	50

4.12 Lipid peroxidation

For lipid peroxidation, fresh tissue (0.5 g FW) was ground to fine powder and extracted in 0.1 % (w/v) trichloroacetic acid (TCA). The supernatant (3 ml) was collected after centrifugation in a test tube and allowed to react with 1 ml of 0.5 % (w/v) thiobarbituric acid in 20 % (w/v) TCA at 100 ^{0}C for 30 min and reaction was terminated on ice. The absorbance at 532 and 600 nm was recorded and MDA contents were expressed as µmol g^{-1} FW using molar extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

4.13 Experimental design and statistical analysis

Experiments were conducted twice with three replicates at a time. Three composite samples were withdrawn for physiological and biochemical analysis. Data was analyzed by one way analysis of variance (ANOVA) using Graphpad 5.1 software. Mean values were compared using Duncan's multiple range test (DMRT) at $P<0.05$ using CoStat 6.4 software (CoHort Software, Pacific Grove U.S.A). *Origin pro 18* software (OriginLab, Northampton, Massachusetts, USA) was used to construct the figures.

5. Results

5.1 *In vitro* screening against drought stress

Osmotic potential of C55 medium supplemented with different concentrations of mannitol were presented in Fig. 5.1. The C55 medium had a osmotic potential of -0.43 MPa. However, the addition of mannitol in the medium resulted in the decline of osmotic potential. This decline was found to be -1.09 MPa at 250 mM mannitol concentration whereas medium supplemented with high concentration of mannitol (1000 mM) had reached a osmotic potential of -3.16 MPa (Fig. 5.1).

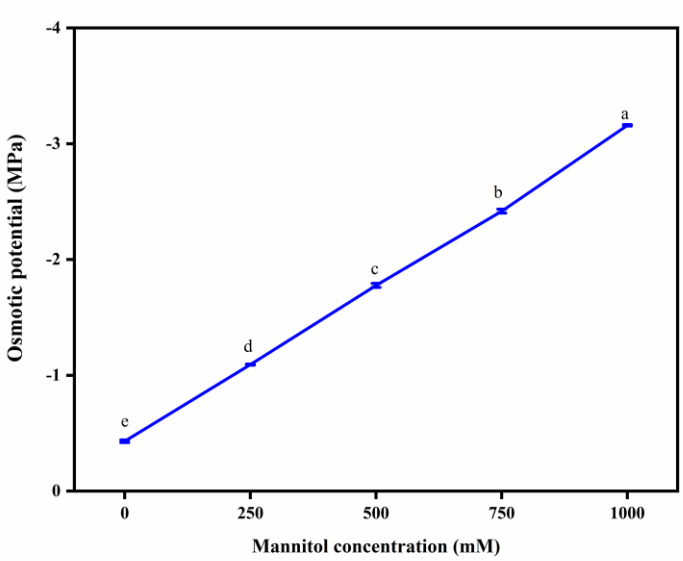

Fig. 5.1 Osmotic potential (MPa) of MS medium supplemented with 2.5 µM benzyl adenine and 0.5 µM α-naphthaleneacetic acid (C55 medium) and different concentrations of mannitol (0-1000 mM). Data were recorded at the beginning of experiment and analyzed by ANOVA. Mean values followed by different letters in the superscript are significantly different at $P<0.05$ by DMRT

5.2 Shoot culture growth index

The multiple shoot clumps of known fresh weight (FW) (*difference between the bottle weight containing medium after inoculation and before inoculation*) were inoculated on medium supplemented with different concentrations of mannitol and incubated at culture conditions for 28 days. When compared to control, the cultures of KE8 remained green for 28 days on various mannitol concentrations whereas browning of the shoot clumps were recorded for 'CE2', 'T1' and 'Y8' clones on medium containing higher concentration of mannitol (≥750 mM) (Fig. 5.2). Fresh weight of the cultures (*difference between the bottle weight after 28 days of incubation and bottle weight containing medium before inoculation*) were recorded after 28 days of incubation and culture growth index of the various clones of *Eucalyptus tereticornis* was calculated at various mannitol concentrations. Culture growth index declined significantly with the increasing concentrations of mannitol (Fig. 5.3). However, in clone 'KE8', culture growth index increased to 247.89 % on medium supplemented with 250 mM mannitol compared to control (236.18 %) (Fig. 5.3). Thereafter, significant decrease of culture growth index was recorded on medium supplemented with high concentration of mannitol (Fig. 5.3). However, mannitol induced drought stress conditions appeared to more harmful to clone 'Y8' than the other two clones ('CE2' and 'T1'). On medium supplemented 1000 mM mannitol, culture growth index of various clones 'KE8', 'CE2', 'T1' and 'Y8' was found to be 141.42 %, 122.23 %, 116.04 % and 106.97 % respectively (Fig. 5.3). The clones were identified as tolerant ('KE8' with culture growth index > 125 %), moderately tolerant ('CE2' and 'T1' with culture growth index 110-125 %), and sensitive ('Y8' with culture growth index <110 %) to drought stress (Fig. 5.2, 5.3).

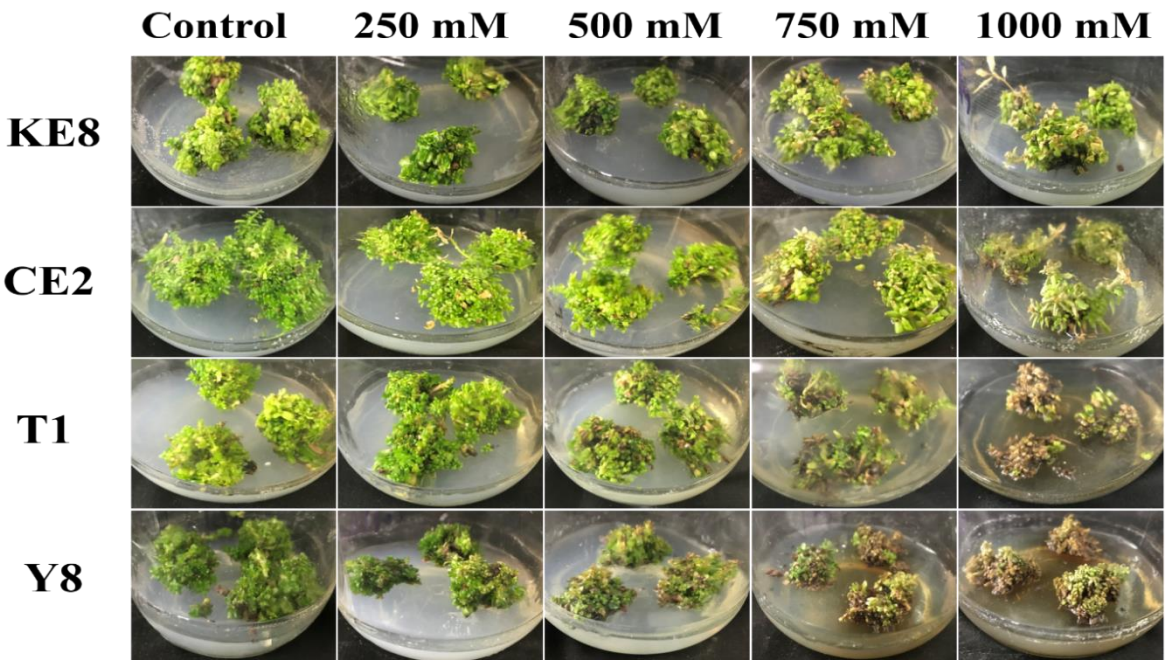

Fig. 5.2 Performance of shoot cultures of selected elite clones of *E. tereticornis* ('KE8', 'CE2', 'T1' and 'Y8') on C55 medium containing different concentrations of mannitol (0-1000 mM) after 28 days of culture.

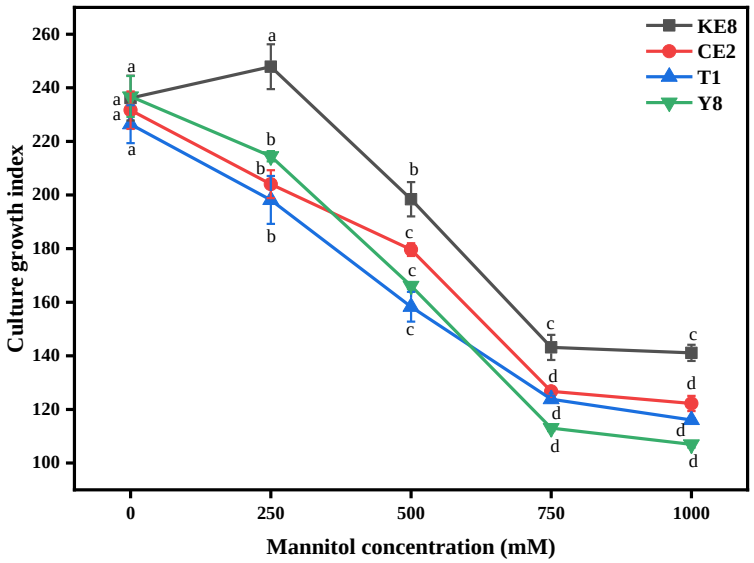

Fig. 5.3 Changes in growth index of shoot culture of different clones of *E. tereticornis* on C55 medium supplemented with different concentrations of mannitol. Data were recorded after 28 days of culture and analyzed by ANOVA. Mean values within series followed by different letters are significantly different at P<0.05 by DMRT.

5.3 Relative water content

Relative water content (RWC) of the various clones was recorded after 28 days of culture on medium containing different concentrations of mannitol (Table 5.1). In the absence of mannitol, RWC was found to be 93.49 % in clone 'KE8' and reduced significantly to 72.79 % on medium supplemented with 1000 mM mannitol. In clone 'Y8', maximum RWC (94.24 %) was recorded on control medium and minimum RWC (60.29 %) was recorded on medium supplemented with 1000 mM mannitol. For other two clones ('CE2' and 'T1'), significant reduction in RWC was also recorded on medium supplemented with 1000 mM mannitol when compared to the control (Table 5.1).

Table 5.1 The effect of various concentrations of mannitol present in C55 medium on relative water content in different clones ('KE8', 'CE2', 'T1' and 'Y8') of *E. tereticornis.*

Mannitol concentration	Clones of *Eucalyptus tereticornis*			
(mM)	KE8	CE2	T1	Y8
0	93.49^{a}	93.36^{a}	96.22^{a}	94.24^{a}
250	89.06^{ab}	87.83^{a}	89.40^{a}	88.59^{a}
500	82.77^{bc}	71.09^{b}	73.47^{b}	67.06^{b}
750	74.69^{cd}	65.58^{b}	64.83^{b}	61.99^{b}
1000	72.79^{d}	63.83^{b}	63.15^{b}	60.29^{b}

Data were recorded after 28 days of culture and analyzed by ANOVA. Mean were compared by DMRT within columns and values followed by different letters in the superscript are significantly different at $P<0.05$.

5.4 Osmotic potential

The significant reduction of osmotic potential was recorded on medium supplemented with different concentrations of mannitol. The osmotic potential of sap was higher (-1.19 to -1.35

MPa) for all the clones on control medium, whereas it was reduced to -3.79 MPa in clone 'Y8' on media containing 1000 mM mannitol after 28 days of culture (Table 5.2). We also recorded that stress tolerant clone 'KE8' maintained higher osmotic potential (-3.30 MPa) when compared with other clones on medium containing 1000 mM mannitol.

Table 5.2 The effect of various concentrations of mannitol present in C55 medium on osmotic potential in different clones ('KE8', 'CE2', 'T1' and 'Y8') of *E. tereticornis*.

Mannitol concentration	Clones of *Eucalyptus tereticornis*			
(mM)	KE8	CE2	T1	Y8
0	-1.32^{a}	-1.35^{a}	-1.19^{a}	-1.31^{a}
250	-2.07^{b}	-2.26^{b}	-1.48^{b}	-1.95^{b}
500	-2.42^{bc}	-2.65^{c}	-2.13^{c}	-2.97^{c}
750	-2.67^{c}	-2.77^{c}	-2.85^{d}	-3.00^{c}
1000	-3.30^{d}	-3.70^{d}	-3.72^{e}	-3.79^{d}

Data were recorded after 28 days of culture and analyzed by ANOVA. Mean were compared by DMRT within columns and values followed by different letters in the superscript are significantly different at $P<0.05$.

5.5 Pigment content

The contents of various pigments (chlorophyll a, chlorophyll b and carotenoid) were also analyzed for all the clones on medium containing different concentrations of mannitol (Fig. 5.4). The increase of mannitol concentration in the medium led to significant decline of pigment contents in various clones of *E. tereticornis.* Maximum chlorophyll A content (114.03 µg g^{-1} FW) for tolerant clone 'KE8' was recorded on medium supplemented with 250 mM mannitol while, minimum chlorophyll A content (91.28 µg g^{-1} FW) was recorded on medium containing 1000 mM mannitol. Similarly, for sensitive clone 'Y8', maximum chlorophyll A content (106.48 µg g^{-1} FW) was recorded on control medium whereas minimum chlorophyll A content (43.77 µg

g^{-1} FW) was recorded on medium containing with 1000 mM mannitol (Fig. 5.4a). Chlorophyll B, total chlorophyll and carotenoid contents also recorded a significant decline in all the clones affecting sensitive clone ('Y8') more than other clones (Fig. 5.4b-d).

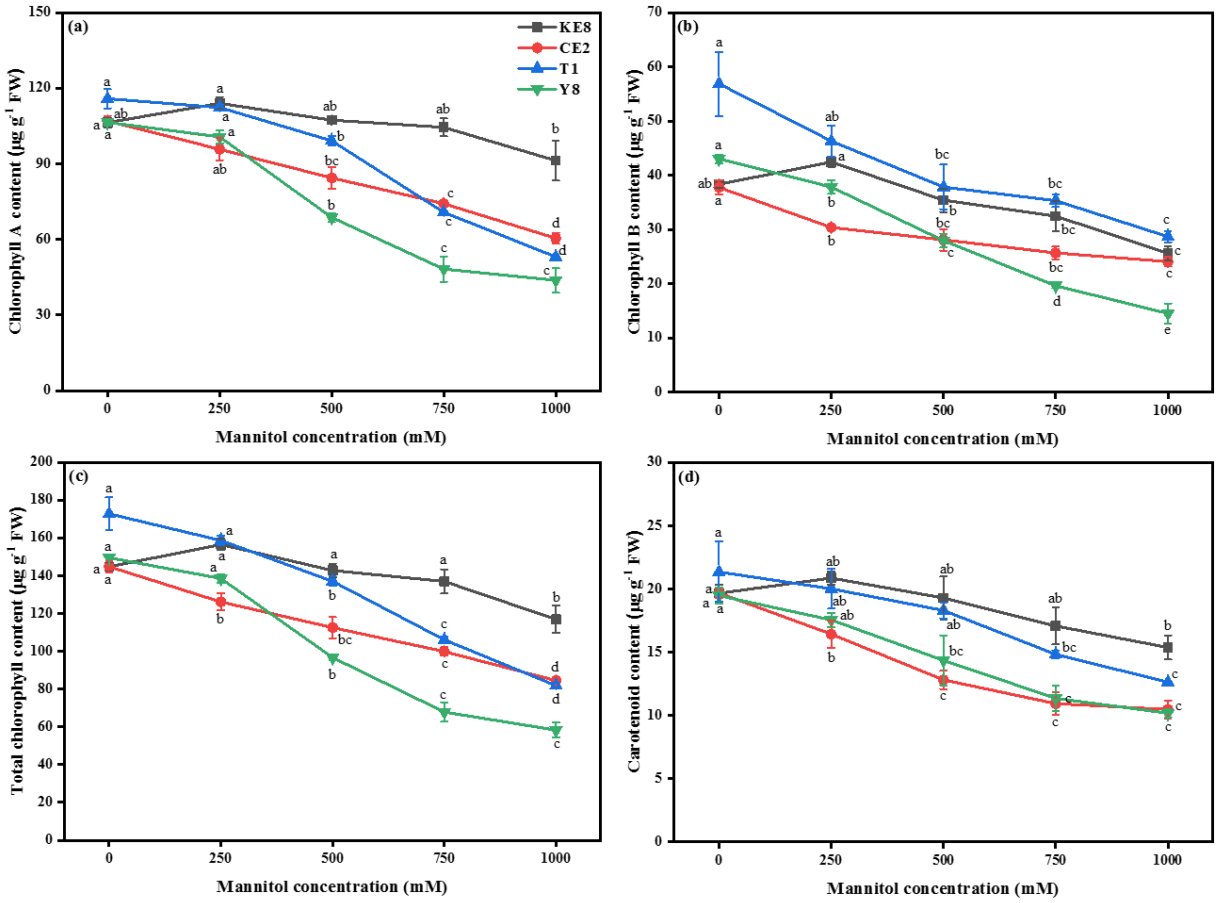

Fig. 5.4 Changes in pigment contents [(a) chlorophyll A; (b) chlorophyll B; (c) total chlorophyll; (d) carotenoid content] in selected elite clones of *E. tereticornis* on C55 medium containing different concentrations of mannitol. Data were recorded after 28 days of culture and analyzed by ANOVA. Mean values followed by different letters within series are significantly different at P<0.05 by DMRT.

5.6 Proline content

In response to drought stress, increase in proline accumulation was recorded in various clones of *E. tereticornis* (Fig. 5.5). Higher proline accumulation (4 µmol g^{-1} FW) was recorded in the cultures of clone 'KE8' on medium containing 750 mM mannitol, whereas lower proline

content (3.2 µmolg^{-1} FW) was recorded for clone 'Y8' on the same medium. With respect to control 2.28, 2.2, 2.04 and 1.74 fold increase in proline accumulation was recorded in the cultures of 'KE8', 'CE2', 'T1' and 'Y8' on medium containing 750 mM mannitol respectively (Fig. 5.5). With the addition of 1000 mM mannitol to the medium, decrease in proline content was observed due to the necrosis of shoot clumps in moderately tolerant ('CE2' and 'T1') and sensitive ('Y8') clones.

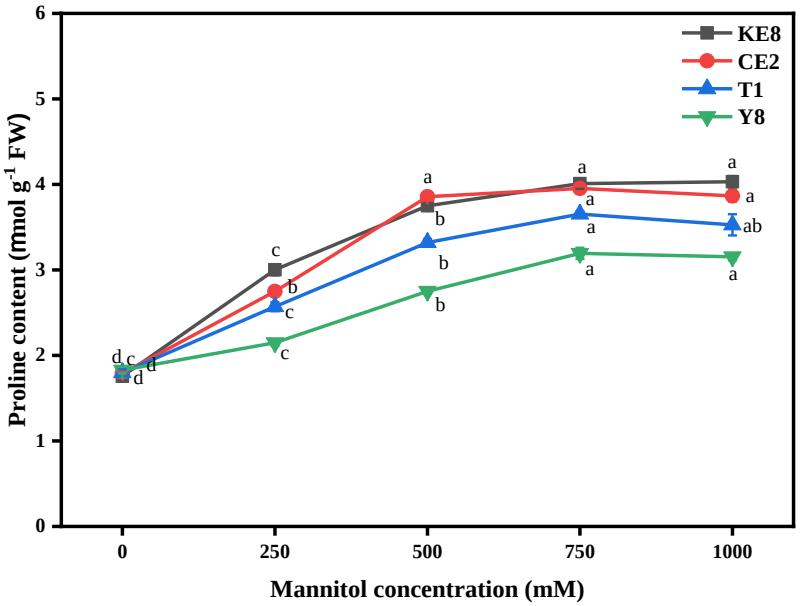

Fig. 5.5 Proline content in different clones of *E. tereticornis* on C55 medium containing different concentrations of mannitol. Data were recorded after 28 days of culture and analyzed by ANOVA. Mean values followed by different letters within series are significantly different at P<0.05 by DMRT.

5.7.1 Total soluble sugar content

Mannitol induced drought conditions resulted in a significant increase in the contents of total soluble sugar in different clones of *E. tereticornis* (Fig. 5.6a). In comparison to control, cultures of tolerant clone 'KE8' recorded maximum total soluble sugar content (169.59 mg g^{-1} DW) on medium supplemented with 1000 mM mannitol, whereas significantly lower content (89.39 mg g^{-1} DW) was recorded in sensitive clone 'Y8' on the same medium.

5.7.2 Reducing sugar content

Reducing sugars increased significantly with increasing concentration of mannitol in the medium (Fig. 5.6b). Maximum reducing sugar content (130.32 mg g^{-1} DW) was recorded in the cultures of clone 'KE8' on C55 medium supplemented with 1000 mM mannitol, whereas the cultures of clone 'Y8' recorded minimum reducing sugar content (79.09 mg g^{-1} DW) on the same medium.

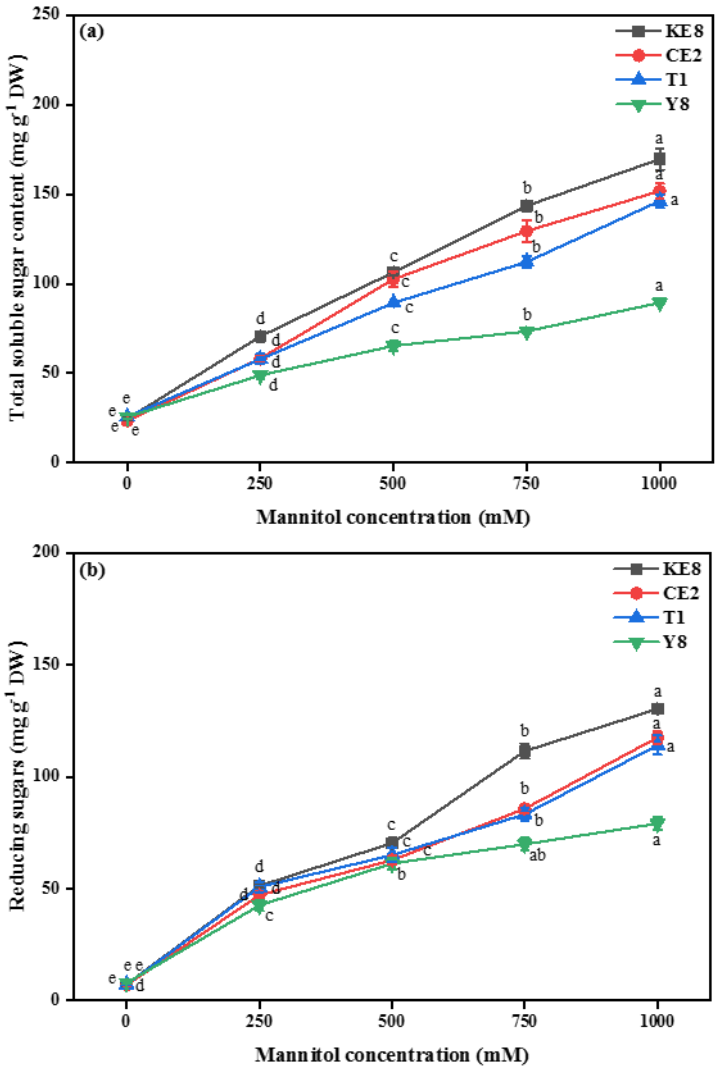

Fig. 5.6 (a) Total soluble sugars and (b) reducing sugars content in different clones of *E. tereticornis* on C55 medium containing different concentrations of mannitol. Data were recorded after 28 days of culture and analyzed by ANOVA. Mean values followed by different letters within series are significantly different at P<0.05 by DMRT.

5.8 Total soluble protein content

Protein content increased significantly in all the clones of *E. tereticornis* on medium supplemented with 250 mM mannitol compared to control (Fig. 5.7). Thereafter, steady increase in protein content was recorded with increasing concentration of mannitol (≤ 750 mM). Protein content was found to be highest (15.03 mg g^{-1} FW) in the cultures of clone 'KE8' on medium supplemented with 1000 mM mannitol, whereas on the same medium, lower protein content (11.64 mg g^{-1} FW) was recorded in the cultures of clone 'T1' (Fig. 5.7).

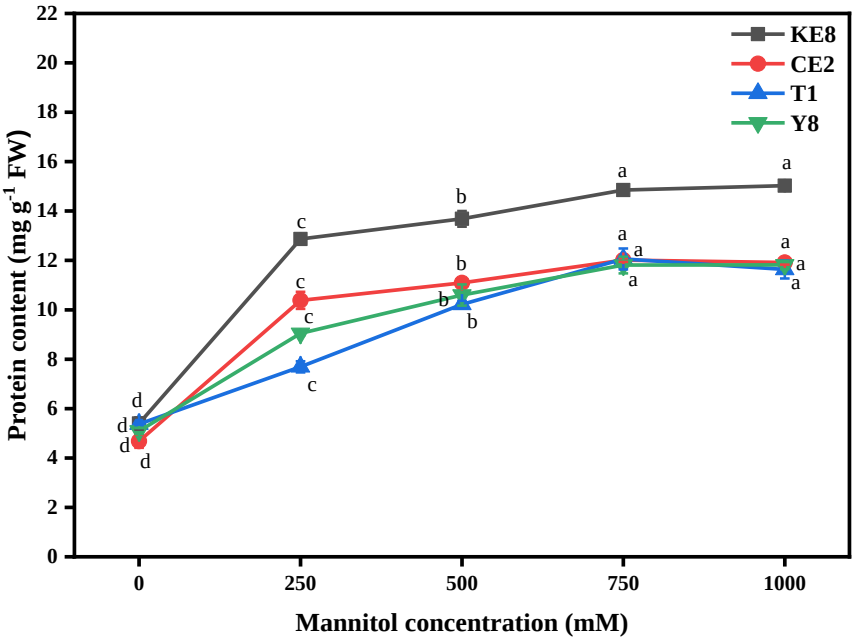

Fig. 5.7 Protein content in different clones of *E. tereticornis* on C55 medium containing different concentrations of mannitol. Data were recorded after 28 days of culture and analyzed by ANOVA. Mean values followed by different letters within series are significantly different at P<0.05 by DMRT.

5.9.1 Superoxide dismutase activity

Superoxide dismutase (SOD) activity was significantly higher (11.09 U mg^{-1} protein) in the cultures of tolerant clone 'KE8' on medium containing 1000 mM mannitol (Table 5.3). On the same medium, the cultures of 'CE2' and 'T1' clones had lower SOD activity of 8.41 and 9.02 U mg^{-1} protein respectively. On the other hand, sensitive clone ('Y8') had maximum SOD activity (7.57 U mg^{-1} protein) on medium containing 500 mM mannitol, while minimum SOD activity (3.06 U mg^{-1} protein) was observed on control medium (Table 5.3).

Table 5.3 The effect of various concentrations of mannitol in C55 medium on superoxide dismutase activity in different clones ('KE8', 'CE2', 'T1' and 'Y8') of *E. tereticornis*.

Mannitol concentration	Clones of *Eucalyptus tereticornis*			
(mM)	KE8	CE2	T1	Y8
0	3.31^{d}	4.09^{c}	3.86^{c}	3.06^{b}
250	4.61^{cd}	4.82^{bc}	5.76^{bc}	4.70^{ab}
500	7.36^{bc}	7.42^{ab}	7.71^{ab}	7.57^{a}
750	9.90^{ab}	8.09^{a}	8.32^{ab}	7.20^{a}
1000	11.09^{a}	8.41^{a}	9.02^{a}	6.99^{a}

Data were recorded after 28 days of culture and analyzed by ANOVA. Mean were compared by DMRT within columns and values followed by different letters in the superscript are significantly different at $P<0.05$.

5.9.2 Catalase activity

Catalase (CAT) showed an increase in the activity under mannitol induced drought conditions recorded after 28 days (Table 5.4). Maximum CAT activity (41.05 U mg^{-1} protein) was observed in the cultures of tolerant clone 'KE8' on medium supplemented with 1000 mM mannitol, while on the same medium, much lower CAT activity (35.12 U mg^{-1} protein) was recorded in the cultures of moderately tolerant clone ('T1'). No significant differences in the CAT activity were observed in the cultures of moderately tolerant clones namely 'CE2' and 'T1' and sensitive clone 'Y8' on medium supplemented with 500 mM or higher mannitol concentration (Fig. 5.4).

Table 5.4 The effect of various concentrations of mannitol in C55 medium on catalase activity in different clones ('KE8', 'CE2', 'T1' and 'Y8') of *E. tereticornis*.

Mannitol concentration	Clones of *Eucalyptus tereticornis*			
(mM)	KE8	CE2	T1	Y8
0	13.64^{c}	17.89^{c}	17.01^{b}	14.95^{c}
250	20.32^{c}	25.67^{bc}	32.34^{a}	29.73^{b}
500	30.42^{b}	33.18^{ab}	37.01^{a}	33.05^{ab}
750	35.55^{ab}	35.07^{a}	36.83^{a}	33.08^{ab}
1000	41.05^{a}	38.54^{a}	35.12^{a}	36.08^{a}

Data were recorded after 28 days of culture and analyzed by ANOVA. Mean were compared by DMRT within columns and values followed by different letters in the superscript are significantly different at $P<0.05$.

5.9.3 Peroxidase activity

Significant variation in peroxidase (POX) activity was recorded among clones of *E. tereticornis* on control medium. For tolerant clone 'KE8', maximum POX activity (300.38 U mg^{-1} protein) was recorded on medium containing 1000 mM mannitol, while minimum POX activity (118.09 U mg^{-1} protein) was recorded in the absence of mannitol (Table 5.5). In sensitive clone 'Y8', POX activity of 114.70 U mg^{-1} protein was recorded on control medium, while increased significantly to 306.99 U mg^{-1} protein on medium containing 1000 mM mannitol.

Table 5.5 The effect of various concentrations of mannitol in C55 medium on peroxidase activity in different clones ('KE8', 'CE2', 'T1' and 'Y8') of *E. tereticornis*.

Mannitol concentration	Clones of *Eucalyptus tereticornis*			
(mM)	KE8	CE2	T1	Y8
0	118.09^{c}	124.74^{c}	154.85^{b}	114.70^{c}
250	155.46^{c}	151.73^{c}	179.48^{b}	174.08^{b}
500	227.62^{b}	201.05^{b}	200.92^{b}	207.07^{b}
750	276.47^{ab}	229.79^{b}	226.03^{ab}	229.19^{b}
1000	300.38^{a}	320.76^{a}	298.14^{a}	306.99^{a}

Data were recorded after 28 days of culture and analyzed by ANOVA. Mean were compared by DMRT within columns and values followed by different letters in the superscript are significantly different at $P<0.05$.

5.9.4 Ascorbate peroxidase activity

Ascorbate peroxidase (APX) activity in the cultures of different clones ('KE8', 'CE2' and 'Y8') of *E. tereticornis* did not differ significantly on control medium and medium containing 250 mM mannitol (Table 5.6). APX activity increased significantly after 28 days of drought stress (1000 mM mannitol) when compared to the control. The cultures of clone 'Y8' exhibited minimum APX activity (434.86 U mg^{-1} protein) on control medium while maximum APX activity (737.33 U mg^{-1} protein) on medium supplemented with 1000 mM mannitol (Table 5.6).

Table 5.6 The effect of various concentrations of mannitol in C55 medium on ascorbate peroxidase activity in different clones ('KE8', 'CE2', 'T1' and 'Y8') of *E. tereticornis*.

Mannitol concentration	Clones of *Eucalyptus tereticornis*			
(mM)	KE8	CE2	T1	Y8
0	390.55^{c}	411.91^{c}	339.06^{c}	434.86^{c}
250	438.61^{bc}	405.92^{c}	487.52^{b}	513.09^{bc}
500	524.55^{b}	589.23^{b}	541.72^{b}	552.63^{b}
750	668.50^{a}	576.64^{b}	547.92^{b}	574.31^{b}
1000	696.44^{a}	716.16^{a}	712.12^{a}	737.33^{a}

Data were recorded after 28 days of culture and analyzed by ANOVA. Mean were compared by DMRT within columns and values followed by different letters in the superscript are significantly different at $P<0.05$.

5.10 Lipid peroxidation

Lipid peroxidation was determined in terms of MDA contents. Maximum MDA content (21.06 µmol g^{-1} FW) was observed in the cultures of clone 'Y8' on medium containing 1000 mM mannitol, while on the same medium, minimum MDA content of 10.03 µmol g^{-1} FW was observed in cultures of clone 'KE8' after 28 days (Fig. 5.8). In other two clones ('CE2' and 'T1'), the MDA content also increased significantly with an increase in mannitol concentration. This clearly indicates that lipid peroxidation is alleviated by increasing the concentration of mannitol in the medium.

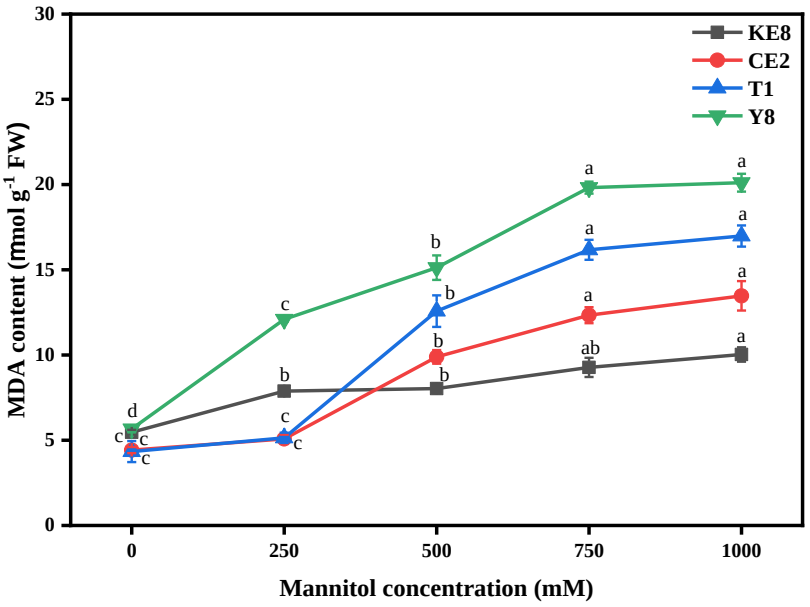

Fig. 5.8 Malondialdehyde (MDA) content in different clones of *E. tereticornis* on C55 medium containing different concentrations of mannitol. Data were recorded after 28 days of culture and analyzed by ANOVA. Mean values followed by different letters within series are significantly different at P<0.05 by DMRT.

6. Discussion

Drought had been considered as multifaceted stress which affects the productivity of the crop. Incidents of drought are rising rapidly in many parts of the world due to poor irrigation and drainage practices from last few decades. In order to cope up with the prevailing situation and looking forward towards sustainable development, selection of drought tolerant plants requires critical attention for plantation under water deficient areas. Selection of plants through *in vitro* cultures exhibiting higher tolerance to drought stress had been elucidated in various commercial crops from past few decades (Bressan et al. 1981; Errabii et al. 2006; Hassan et al. 2004; Mohamed et al. 2000).

However, evaluation of crops under field conditions are highly dependent upon the variability of weather and unpredictable climatic conditions, whereas *in vitro* screening can be performed in a homogenous and controlled manner for the selection of drought tolerant clones. The general effect of drought on the *in vitro* raised plantlets using different stress inducing agents (mannitol, sorbitol, polyethylene glycol, sodium chloride etc.) had been earlier reported in many plants like potato (Gopal and Iwama 2007), barley (Hellal et al. 2018), wheat (Ji et al. 2014b), sugarcane (Rao and Jabeen 2013).

In present study, *in vitro* screening of *E. tereticornis* clones for drought tolerance was carried out using mannitol as the stress inducing agent (Fig. 5.2). Mannitol is used as a carbon source and reduced the water potential of the culture medium (Lipavska and Vreugdenhil 1996). The use of mannitol for drought tolerance under *in vitro* conditions had been reported earlier in various plant species such as sugarcane (Errabii et al. 2006), *Brassica juncea* (Gangopadhyay et al. 1997), Mexican marigold (Mohamed et al. 2000), populous (Watanabe et al. 2000). In present study, selected elite clones of *E. tereticornis* when cultured on medium supplemented with increasing concentrations of mannitol, showed significant differences in the cultures based on the culture growth index calculated after 28 days (Fig. 5.3). Growth inhibition was higher in sensitive clone ‘Y8’ as cultures turned brown on medium supplemented with 1000 mM mannitol. The results are in line with published reports which highlighted shoot growth reduction under drought stress conditions and it was more pronounced in sensitive clones than the tolerant clones (Errabii et al. 2006; Kaya et al. 2013; Rao and Jabeen 2013).

Data from the present study highlighted significant reduction in RWC with increasing concentrations of mannitol (Table 5.1). RWC of tolerant clone was significantly higher on medium containing 1000 mM mannitol than the moderately tolerant and sensitive clones. In order to understand the dehydration tolerance, RWC is widely used as a parameter to study the water status of the plant tissue. In context to the previous findings, our results are in agreement with sugarcane (Errabii et al. 2006) and rice (Hsu and Kao 2003) which showed reduction in RWC with increasing drought stress.

In present study, tolerant clone 'KE8' recorded decrease in osmotic potential with the increasing concentrations of mannitol also in the medium whereas, highest reduction was recorded on medium containing 1000 mM mannitol, indicating the growth reduction under drought conditions (Table 5.2). The decrease in osmotic potential is considered as a protective mechanism to maintain the osmotic balance and plant growth under stressed conditions (Sakthivelu et al. 2008). Previous studies also recorded the decrease of osmotic potential with increasing drought stress in wheat, potato and rapeseeds (Lipavska and Vreugdenhil 1996).

In the present study, it was recorded that with increasing the mannitol concentration, pigment contents also showed a significant reduction in various clones of *E. tereticornis* (Fig. 5.4). Results of the present study also highlighted that sensitive clone had lower chlorophyll and carotenoid contents than the tolerant clone and vice-versa. In addition, the tolerant clone 'KE8' showed an increase in chlorophyll and carotenoid content when cultured on medium containing 250 mM mannitol which can be directly correlated with an increase in culture growth index, however no significant difference was observed when compared to the control. These results are in agreement with the already published reports highlighting reduction in chlorophyll and carotenoid content with increasing drought stress (Jaleel et al. 2009; Shivakrishna et al. 2018).

Proline accumulation was recorded in culture with increasing concentration of mannitol (Fig. 5.5). Therefore, 'KE8' being a tolerant clone and able to tolerate high mannitol concentration recorded a significant higher proline accumulation, as compared to sensitive clone 'Y8'. Proline accumulation is recognized as osmoprotectant under water deficient conditions and used as a marker for water stress (Farooq et al. 2009). These results are in agreement with previously published reports on *Populous* (Watanabe et al. 2000) and Mexican marigold

(Mohamed et al. 2000), which reported proline accumulation was more pronounced in tolerant clone. Further, it was also reported that selected drought tolerant calli of *Brassica* accumulated proline by several folds higher proline than that of control (Gangopadhyay et al. 1997). Similarly, in another study, increased proline accumulation by 10 to13 folds had been reported in sugarcane on medium containing 300 mM mannitol concentration (Errabii et al. 2006).

In the present study, total soluble sugar content also increased significantly with increasing drought stress, however increase was more pronounced in tolerant clone than moderately tolerant and sensitive ones (Fig. 5.6b). Studies conducted on Mexican marigold (Mohamed et al. 2000) reported that soluble sugar increased significantly when exposed to drought conditions. This clearly indicated that plant adjust the osmotic potential by increasing its sugar levels in response to stress conditions.

The present study also highlighted significant increase in protein content with increasing drought stress (Fig. 5.7). The protein content may increase, probably due to the production of stress related proteins in response to mannitol induced stress conditions. However, decline in protein content in sensitive clone may be associated with oxidative damage due to the generation of free radicals. These results are in corroboration with the earlier studies on wheat cultivars and *Centaurea ragusina* (Radić et al. 2006; Ullah et al. 2014) reporting increase of protein content in tolerant clone, whereas reduction in sensitive clone.

In the present study, activity of superoxide dismutase (SOD) increased significantly in tolerant clone with increasing drought stress than sensitive clone (Table 5.3). Study also highlighted that activity of various antioxidant enzymes were strongly influenced in the tolerance level and concentration of mannitol (drought inducing agent) present in the medium (Table 5.3, 5.4, 5.5, 5.6). Increased antioxidant enzyme activity under drought stress is reported to play a significant role in fighting stress tolerance in plants (Gopal and Iwama 2007; Kaya et al. 2013). It is well established that SOD enzyme participate in the scavenging of superoxide radicals and acts as a first component to activate the transduction pathway of antioxidative defense system. These results are in line with the findings, which reported increase in SOD activity with increasing drought stress (Hellal et al. 2018; Hsu and Kao 2003; Rao and Jabeen 2013).

Similarly, the activities of CAT, POX and APX also increased significantly with increasing concentration of mannitol in culture medium (Table 5.4, 5.5, 5.6). These enzymes participate in the elimination of hydrogen peroxide by converting it into water and oxygen. In the present study, significant increase in the antioxidant enzyme activity was observed which are in association with earlier reports on maize (Kaya et al. 2013; Seckin et al. 2009).

In present study, it was observed that MDA content of sensitive clone was significantly higher than that of tolerant clone under stress conditions (Fig. 5.8). Higher levels of MDA content indicated increased lipid peroxidation which in turn is related with more sensitivity for drought stress whereas in tolerant clone, lower levels of MDA content reported to represent less oxidative damage and more tolerance to drought stress (Sairam et al. 1998). In context to the findings on lipid peroxidation in response to various stress inducing agents, the results of present study are in line with published reports in C. *ragusina* plants (Radić et al. 2006) and wheat roots (Seckin et al. 2009), causing a significant increase in MDA contents in sensitive clones.

7. Conclusion

Variation in drought tolerance was examined under *in vitro* conditions using mannitol as stress inducing agent and this study depicted strong variability in drought tolerance among selected elite clones of *Eucalyptus tereticornis.* The clone 'KE8' tolerate upto 1000 mM mannitol whereas sensitive clone 'Y8' tolerate only upto 500 mM mannitol present in the medium after a culture period of 28 days. The other two clones 'CE2' and 'T1' also tolerate upto concentration of 750 mM of mannitol present in the medium. Further, growth inhibition under mannitol induced drought conditions can be directly correlated with reduction in pigment contents, relative water content, osmotic potential, accumulation of various osmolytes, increase of antioxidant enzyme activity and lipid peroxidation. The results of the present study indicated strong association of physiological and biochemical parameters with drought tolerance. Hence, tolerant clone 'KE8' can be considered suitable candidate for plantation under water deficient areas.

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Annexure I

Composition of Murashige and Skoog medium

Macronutrients (mg/l)

NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170

Micronutrients (mg/l)

$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2Fe-EDTA	30

Vitamins (mg/l)

Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo – inositol	100

Sucrose	30g/l
Agar-agar	7g/l

Annexure II

1. Chilled 80 % (v/v) acetone

Prepared by adding 80 ml acetone in 20 ml distilled water and stored at 4 ºC.

2. 2.5 % (w/v) acid ninhydrin solution

Prepared by dissolving 1.25 g ninhydrin in 50 ml glacial acetic acid, with agitation until dissolved.

3. 3 % (w/v) aqueous sulfosalicyclic acid

Prepared by dissolving 3 g of sulfosalicyclic acid in 100ml distilled water.

4. 80 % (v/v) ethanol

Added 80 ml ethanol to 20 ml distilled water.

5. 90 % (v/v) phenol solution

Prepared by dissolving 900 µl phenol in 100 µl distilled water.

6. Dinitrosalicyclic acid (DNSA)

Prepared by dissolving 2.5 g of DNSA in 50 ml distilled water. Also, 4 g NaOH and 75 g disodium tartarate were added and dissolved. Total volume was made up to 250 ml with distilled water.

7. 50 mM potassium phosphate buffer (pH 7.0)

Dissolved 4.672 g of K_2HPO_4 (2.48 mM) and 3.154 g of KH_2PO_4 (2.52 mM) to 800 ml distilled water and final volume was made to 1000 ml. pH was adjusted to 7.0 (if required).

8. 50 mM potassium phosphate buffer (pH 6.8)

Dissolved 3.978 g of K_2HPO_4 (2.28 mM) and 3.696 g of KH_2PO_4 (2.72 mM) to 800 ml distilled water and final volume was made to 1000 ml. pH was adjusted to 6.8 (if required).

9. 0.1N NaOH

Prepared by dissolving 0.4 g NaOH in 100 ml distilled water.

10. 2 % (w/v) Na_2CO_3 solution

Prepared by adding 2 g of Na_2CO_3 in 100 ml 0.1N NaOH.

11. 0.5 % (w/v) $CuSO_4.5H_2O$

Prepared by dissolving 1 g disodium tartarate and 0.5 g $CuSO_4.5H_2O$ in 80 ml distilled water and final volume was made to 100 ml.

12. 1N Folin Reagent

Prepared by diluting 10 ml folin ciocalteu phenol reagent with 10 ml of distilled water.

13. 20 % (w/v) Trichloroacetic acid (TCA)

Prepared by dissolving 10 g TCA in 40 ml of distilled water and final volume was made to 50 ml.

14. 0.1 % (w/v) TCA

Prepared by dissolving 0.1 g of TCA in 100 ml distilled water.

26. **0.5 % (w/v) Thiobarbituric acid (TBA)**

Prepared by dissolving 0.125 g of TBA in 10 ml of 20 % TCA and total volume was made up to 25 ml with 20 % TCA.

15. 1 mM Nitro blue tetrazolium (NBT)(Mol wt :817.64 g/mol)

Prepared by dissolving 8.17 mg NBT in 10 ml distilled water.

16. 1 M L. Methionine (Mol wt :149 g/mol)

Prepared by dissolving 1.49 g of L. Methionine in 10 ml distilled water.

17. 3 mM Ethylenediaminetetraacetic acid (EDTA) disodium salt (Mol wt : 372.24g/mol)

Prepared by dissolving 11.16 mg EDTA disodium salt in 10 ml of distilled water (pH adjusted to 8.0).

18. 0.2 mM Riboflavin (Mol wt : 376 g/mol)

Prepared by dissolving 0.752 mg of riboflavin in 10 ml distilled water.

19. 1 % w/v O-dinisidine solution

Prepared by dissolving 50 mg O-dinisidine in 5 ml of methanol.

20. 10 mM EDTA (Mol wt : 292.25 g/mol)

Prepared by dissolving 0.292 g of EDTA in 100 ml distilled water (pH adjusted to 8.0).

21. 100 mM Ascorbate (Mol wt : 176.12 g/mol)

Prepared by dissolving 1.76 mg of ascorbate per ml of distilled water.

Thesis

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